Localized, Internal, and Supramolecular Polyuronide Motions in Cell Wall Matrices: A Comparison of Solid-State NMR and EPR Relaxation Behavior†

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Previous work has revealed that spin-labeled cell wall homopolygalacturonans display internal motions predominantly about the polymer's main chain. In this work we show that the nitroxyl amide's relaxation behavior is modulated by these same motions. Polyuronide-bound $^{23}Na^+$ spin–lattice relaxation times as well as $^{13}C{=}O$ rotating frame T_1s, which are tempered by the *local* carboxyl group environment, of non-spin-labeled, but otherwise identical, apple tissue manifested a 15–19% decrement as a function of differing levels of tissue ripeness. Similarly, a *higher-order* or supramolecularly averaged measure of cell wall polyuronide motion, C=O-associated T_{1H}, declined 63% with tissue ripeness. In paramagnetically labeled treatments the nitroxyl amide's relaxation parameter diminished ca. 49%. The scope of these molecular level perturbations follows the order local < internal < supramolecular and argue that the changes in polygalacturonan flexibility associated with ripening result from processes other than the cleavage of the polyuronide backbone.

INTRODUCTION

The cell wall and middle lamellar complex of apple fruits is a structurally complicated (Darvill et al., 1980), mostly polysaccharide, solid matrix which is agriculturally important because it affects tissue structure and, therefore, such intransigent macrorheological properties as flesh firmness or crispness. The primary cell wall of most higher plants consists of an infrastructure of cellulose microfibrils held together by a rigid, gellike, lattice of matrix polysaccharides (Preston, 1974) which extend into regions between cell walls known as the middle lamella (Darvill et al., 1980). The matrix polysaccharides, which make up approximately two-thirds of the total apple tissue mass, are hydrophilic, polyhydroxy macromolecules which are extensively hydrated in vivo. In apple fruits, one of these matrix polysaccharides, known as pectin, is based on linear blocks, ca. 40–80 monomer units long (Irwin et al., 1988), of α-(1→4)-linked D-galacturonic acid interspersed with (1→2)- and (1→2 or 4)-substituted L-rhamnose, which is usually further linked with neutral polymers such as arabinans and arabinogalactans, forming side chains. Recent work (Renard et al., 1991) has suggested that these neutral sugar side chains, or "hairy" regions (de Vries et al., 1986), of protopectin (the insoluble form) are principally unconnected to any other cell wall polymer via glycosidic linkages. Further befouling the already complex molecular picture is the fact that the C_6 carboxyl groups of apple pectin are highly esterified (Irwin et al., 1985a) and these methyl ester functionalities may even reside blockwise (Jarvis, 1984; Irwin et al., 1988) in hydrophobic domains.

The ripening or flesh-softening process in apples is physically interesting because it clearly involves the dissolution (Ben-Arie et al., 1979) of the middle lamella yet the molecular level agents behind this process are not completely understood. In most climacteric fruit, such as pears, the disruption of the middle lamella is synchronous with increasing hydrolytic enzyme activity, particularly random-cleaving polygalacturonase (Knee, 1978a,b, 1982a,b; Bartley et al., 1982), which would necessarily solubilize these important matrix polysaccharides. In apples no polygalacturonase has been successfully isolated (Knee, 1982b), the ostensible molecular weight of soluble (Knee, 1978a) pectin in the apple matrix does not appear to change, and there is effectively no measurable transformation in the degree of the methyl esterification (Knee, 1978b; Irwin et al., 1985a) coincident with this dissolution process. Thus, the cell wall "glue", pectin, that compound making up a great deal of the middle lamella (Darvill et al., 1980), appears to become more soluble without concomitant changes in its apparent molecular weight. We utilize the term apparent because polyuronides, like many cell wall polysaccharides, are known (Davis et al., 1980; Fishman et al., 1984, 1986, O'Beirne and Van Buren, 1983) to form extended arrays in aqueous solution and, therefore, the determination of true molecular weights can prove to be elusive due to intermolecular aggregation.

Applications of magnetic resonance technology to problems of agricultural interest provide unique information about the structure of heterogeneous solid matrices. Since many of the mechanical properties of aggregated polymeric materials can be related to chain dynamics and localized molecular motions (Havens and Koenig, 1983; Schaefer and Stejskal, 1979; Yannoni, 1982) we have chosen to

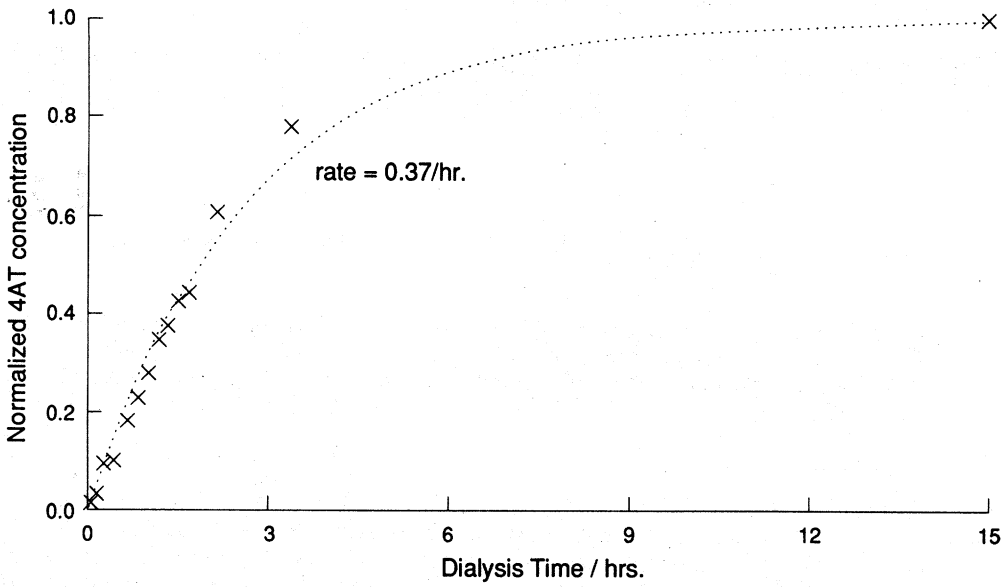

Figure 1. Equilibrium dialysis of 4AT into a 60% ethanol/water milieu of suspended powdered apple cell wall matrices at 25 °C. The final concentration of the entire solution was 2.26×10^{-3} M.

extend previous work (Chamulitrat et al., 1988; Chamulitrat and Irwin, 1989; Irwin et al., 1984a, 1992) on apple cortical cell walls using the inhomogeneous EPR saturation curve technique (Eaton and Eaton, 1990; Metz et al., 1990; Matthys et al., 1988) to observe perturbations in localized, main-chain, and higher order molecular motions as a function of various processes related to the dissolution of the middle lamella.

MATERIALS AND METHODS

Apple Tissue Preparation and Dehydration. As described heretofore (Chamulitrat et al., 1988; Chamulitrat and Irwin, 1989; Irwin et al., 1984a, 1985a), Golden Delicious apple fruits were obtained from the Beltsville Agricultural Research Center. Fruits were harvested, randomized, and stored immediately at approximately 0 °C in 1% (v/v) O_2. After 1 month, fruits were removed and allowed to ripen 0, 11, and 21 days at 20 °C in controlled environment of humidified and ethylene-scrubbed air. At each time interval, 10 fruit were randomly selected and firmness was measured (Irwin et al., 1984a). The fruit were peeled, cut up into ca. 5 × 5 × 3 mm pieces, dipped into 10 mM $CaCl_2$(aq) to inhibit enzymatic tissue oxidation, and subsequently vacuum infiltrated for 2 h or more consecutively in 20, 40, 60, and 80% (v/v) ethanol/water. The partially dehydrated tissue was then equilibrated in 95% ethanol/water for a minimum of 1 day with repeated solution replacement. At this stage samples to be utilized for spin-labeling experiments were ground to a powder in 60% ethanol/water as described formerly (Chamulitrat and Irwin, 1989). Samples for solid-state NMR analysis were further exchanged in absolute ethanol and critical point dried (Irwin et al., 1984a).

Polyuronide Spin-Labeling Conditions and Treatments. Uronide nitroxyl amide (Evelyn and Hall, 1979) reactions were performed as described elsewhere with relatively minor modifications (Chamulitrat et al., 1988; Chamulitrat and Irwin, 1989; Irwin et al., 1987). To minimize spin label reaction heterogeneity, approximately 1.25 g of the 95% ethanol/water-fixed apple tissue was ground to a fine powder in 50 mL of 60% ethanol/water and placed in a 30-cm (≥1200 molecular weight cutoff) Sigma dialysis "sack" (Lot 50F-6107). Representative samples from 0, 11, and 21 days poststorage were all processed as above and their dialysis sacks collectively suspended in 60% ethanol/water (pH ca. 4.75) with gentle agitation thereafter. The total volume of this suspension was ca. 450 mL. As shown earlier (Chamulitrat and Irwin, 1989), this solvent system was utilized to avoid the partial solubilization of the cell wall matrix polysaccharides as could occur in water. Approximately 174 mg of 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (4AT) was added to the suspension, and the pH was readjusted to starting conditions and maintained at this pH thereafter. After 2 h, the approximate time it takes for about half of the 4AT to diffuse into each dialysis sack (Figure 1), ca. 90 mg of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) was added to the reaction mixture slowly over 2-h intervals, to a total of ca. 0.8 g, over 48 h. Upon stabilization of the pH, the reaction mixture was adjusted to neutrality, and the reaction, activated ester → nitroxyl amide (Taylor and Conrad, 1972), was continued for an additional 72 h. The samples were then washed, dehydrated, and critical point dried as described elsewhere (Chamulitrat and Irwin, 1989). Poly(galacturonic acid) (Sigma H^+ form; PGA) nitroxyl amide spin-labeling reactions were performed precisely as described previously (Chamulitrat et al., 1988). Regarding the Ca^{2+}–PGA experiments, 20 μL of an 1% (w/v) nitroxyl amide-labeled polyuronide solution was injection precipitated into 10 mL of 60% EtOH/H_2O with enough $CaCl_2$ to make a 1 mM solution and resulted in <20% of the binding sites being filled (Chamulitrat and Irwin, 1989). The Ca^{2+}–PGA samples were subsequently powdered after exchanging with absolute ethanol and vacuum drying. The day 0 cell wall sample was hydrated [ca. 60% (w/w)] as described formerly (Chamulitrat and Irwin, 1989).

Spectroscopic Measurements. All solid-state NMR and EPR experimental conditions and calculations have been published earlier (Irwin et al., 1984a, 1985a; Chamulitrat et al., 1988; Chamulitrat and Irwin, 1989). The number of nitroxyl amide spins for each sample ($\bar{x} = 5.35 \times 10^{18} \pm 7\%$ spins/g of dry weight) was calculated against a rodlike Cr^{3+}/Al_2O_3 crystal (8.99×10^{15} spins).

RESULTS AND DISCUSSION

Because of the relative ease and specificity of "labeling" pectin's C_6 carboxyl group, we have designated this functional domain to attach various "reporter" groups such as $^{23}Na^+$ (Irwin et al., 1984a), Mn^{2+} (Irwin et al., 1984b, 1985b), and Cu^{2+} (Irwin et al., 1985a,b), and 4AT reacted as the nitroxyl amide (Chamulitrat et al., 1988; Chamulitrat and Irwin, 1989; Irwin et al., 1987, 1988). Ultimately, this work has been performed to answer questions about the submolecular behavior of sugar acid-containing polymers which abut highly diverse microenvironments. This diversity of surroundings exists because any particular pectinic polyuronide block is enfolded within a lattice of analogous polyanions as well as neutral heteropolymers. Since perturbations in the three-dimensional architecture enveloping pectin could influence its microenvironment, and therefore molecular dynamics, we have chosen to extend previous work (Irwin et al., 1984a) on apple cortical

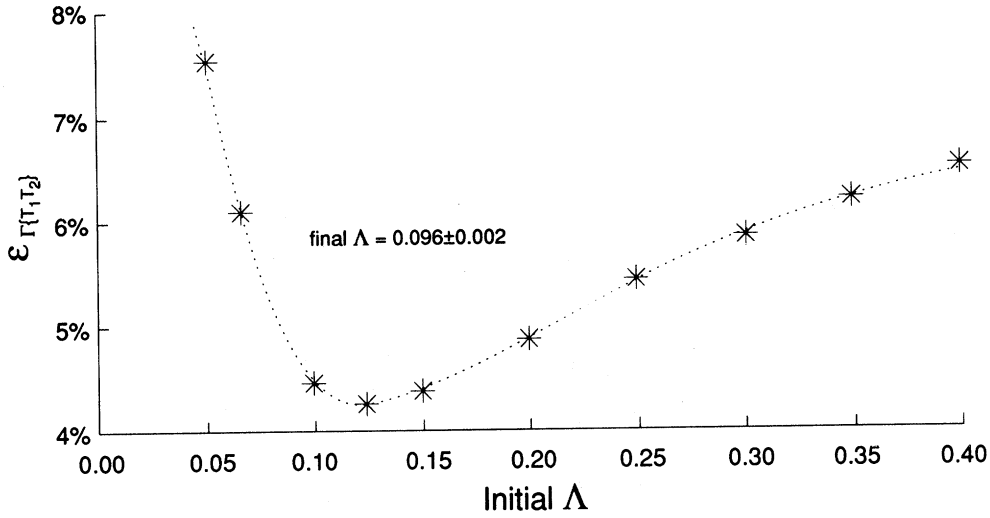

Figure 2. Dependence of $\Gamma\{T_1T_2\}$-associated standard error on initial estimates of T_{2*}/T_2.

cell walls using the inhomogeneous EPR saturation curve technique (Eaton and Eaton, 1990; Metz et al., 1990; Matthys et al., 1988) so as to scrutinize local (specific to the region adjacent to or at the C_6 carboxyl group; <10 Å), internal (within the polyanionic blocks of pectin; ca. 10–100 Å), and supramolecular (at the lattice or aggregate level; >100 Å) polymeric dynamics as a function of various degrees of middle lamella dissolution. We have studied these processes (Irwin et al., 1984a) indirectly by the determination of various spin–lattice relaxation times, which are, for amorphous solids, inversely proportional to molecular motion if one assumes that all other contributing factors (e.g., spin concentration and distribution) remain constant within the boundaries of the comparisons being made (Abragam, 1986; Eaton and Eaton, 1990; Havens and Koenig, 1983; Schaefer and Stejskal, 1979; Yannoni, 1982).

Relationship between EPR Power Saturation Curves and Internal Nitroxyl Amide Motions. For this work the relaxation parameter, $\Gamma\{T_1T_2\}$, was calculated as described by Metz and co-workers (Metz et al., 1990). Using this method the EPR first-derivative intensity (inhomogeneously broadened Lorentzian line shape), I', for any one of the nitroxyl amide transitions ($m = -1$, 0, or 1) has been described as a function of the arguments Ψ, $\Gamma\{T_1T_2\}$, and Λ as

$$I' = \frac{\Psi(\Pi_\mu)^{1/2}}{\{1 + \Gamma\Pi_\mu\}^{1/2}} \frac{1}{\{1 + \Lambda\{1 + \Gamma\Pi_\mu\}^{1/2}\}^2}$$

where

$$\Psi = \frac{I_0' B_1}{(\Pi_\mu)^{1/2}} \qquad \Gamma\{T_1T_2\} = \frac{\gamma^2 B_1^2 T_1 T_2}{\Pi_\mu} \qquad \Lambda = \frac{T_{2*}}{T_2}$$

and Π_μ, the microwave power (in watts), is the x variable. Please note that the above equation was incorrectly reported (Metz et al., 1990) to not have the square root of Π_μ dependency in the numerator; we acknowledge the astute reviewer who noticed this error. In this relationship I_0' is the maximum I' and B_1 is the rotating magnetic field perpendicular to the large static field B_0. All other terms have their usual meaning (Poole, 1983; Wertz and Bolton, 1986). Using a curve fitting algorithm, such as the Gauss–Newton method (Draper and Smith, 1981) for fitting nonlinear regression functions by least-squares analysis, one can iteratively solve for all of the above parameters. In practice, however, we found that we could minimize the error in the Γ term by first fixing Λ at 0.125 (Figure 2) and solving for all remaining terms. All of the calculations presented in this paper used, as initial estimates, ca. 2×10^9 for Ψ and 1000–3000 for $\Gamma\{T_1T_2\}$ with Λ fixed at 0.125. The final estimate of Λ for all tested treatments was 0.096 ± 0.002. The latter finding, that Λ or $T_{2*}/T_2 \ll 1$, is evidence that these spin-labeled polyuronide systems undergo complete inhomgeneous saturation (Metz et al., 1990).

Another paramagnetic spin–lattice relaxation parameter, T_1T_{2*}, can also be estimated (Figure 3) from Π_μ at the point of maximum $I'(\Pi_\mu^{max})$ since T_1T_{2*} is inversely proportional to Π_μ^{max}. For the Ca^{2+}-treated PGA sample the Π_μ^{max} was ca. 22.18 mW (the square of that value determined where $\partial I'/\partial(\Pi_\mu)^{1/2} = 0$) while that of the control was ca. 34.68 mW, indicating that the relative Ca^{2+}–PGA T_1s, as defined above, were approximately 56% larger than those of the control PGA treatment. Fitting the growth phase of the power saturation data to the Metz equation (Figure 4) yields a T_1-related parameter, $\Gamma\{T_1T_2\}$, which changes, as a function of treatment (e.g., Ca^{2+} $\Gamma\{T_1T_2\}$ 35% > control), similar to the T_1T_{2*} parameter calculated above. This result is not unexpected since, for similar samples in the solid state, T_{2*} and T_2 should change less than T_1 (Abragam, 1986). These samples are a good test for the power saturation method, described above, since we have previously noted (Chamulitrat and Irwin, 1989) that the energy of activation (E_a) for main-chain, or "internal", motions for these Ca^{2+} salts enlarged 50% (4.2 to 6.3 kcal/mol) when the amount of Ca^{2+} bound was increased from 20 to ca. 40% of the binding sites filled, thereby indicating that Ca^{2+} cross-linking restricts the internal motions of the polyuronide chains. These data argue that EPR steady-state microwave power saturation curves are modulated by pectin's internal, or main-chain, motions.

Power saturation curves (Figure 4) for control (day 0) spin-labeled apple cortical tissue in the dry ($\Gamma\{T_1T_2\}$ = 3104) and hydrated ($\Gamma\{T_1T_2\}$ = 2015) solid state show that our spin–lattice relaxation parameter decreased, as one would expect (Abragam, 1986), when the overall molecular motion was increased via hydration. Figure 5 (inset) also shows a Carr–Purcell–Meiboom–Gill (Farrar, 1987) ^1H (60-MHz) NMR experiment on a comparably hydrated sample, the paramagnetic amide of which had been previously reduced to the N–OH form (Irwin et al., 1987). In this experiment, the semilog plot of normalized resonance

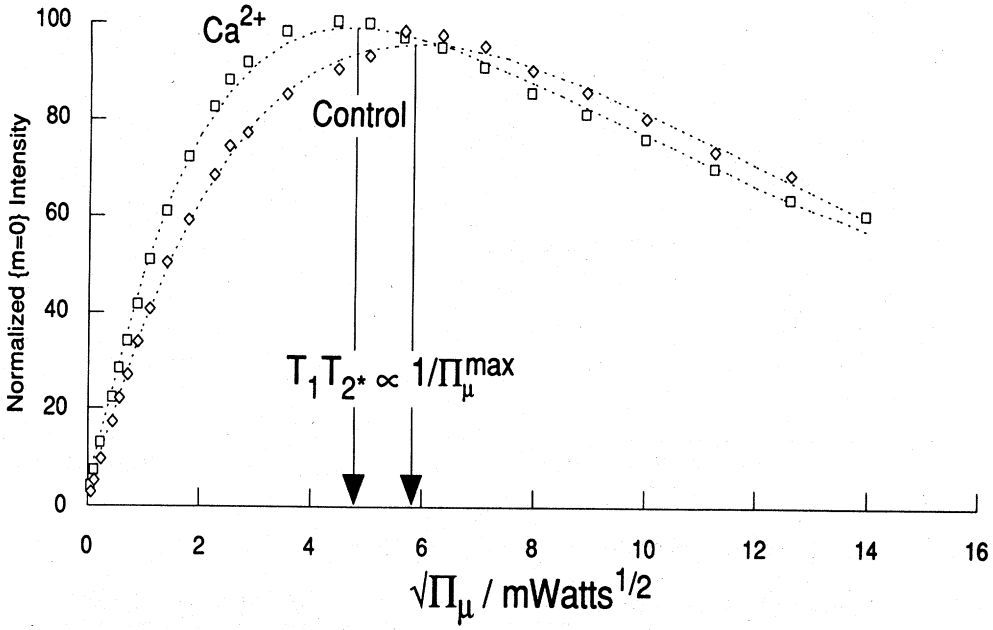

Figure 3. Plot of the total power saturation curves, first derivative $m = 0$ transition vs $(\Pi_\mu)^{1/2}$, of nitroxyl amide-labeled PGA and its, partial, Ca^{2+} salt. A paramagnetic spin–lattice relaxation parameter, T_1T_{2*}, was estimated from Π_μ at the point of maximum intensity since $T_1T_{2*} \propto 1/\Pi_\mu^{max}$. For the Ca^{2+}-treated PGA sample, the Π_μ^{max} was ca. 22.18 mW (the square of that value determined where $\partial I'/\partial(\Pi_\mu)^{1/2} = 0$) while that of the control was ca. 34.68 mW.

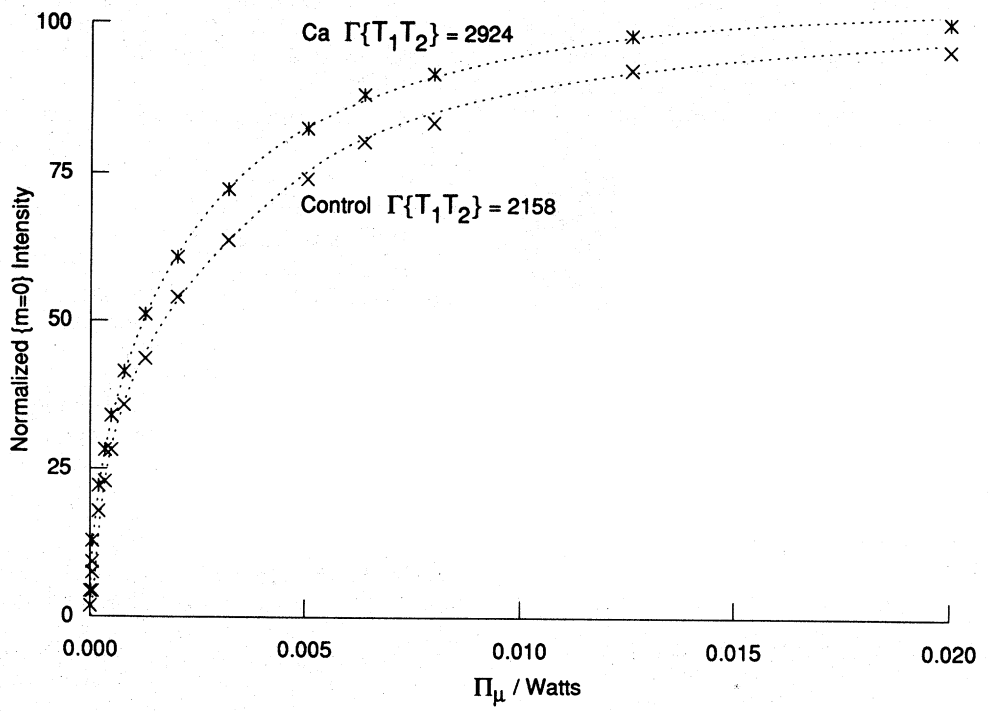

Figure 4. Partial power saturation curves of nitroxyl amide-labeled PGA and its Ca^{2+} salt fit to the Metz function.

integrals, as a function of the variable delay, 2τ, clearly demonstrates two populations of spin–spin relaxation. The y intercept for the slowly relaxing H_2O population (fast motion) was utilized as a relative measure of the activity of "free" H_2O presumably located in an outer hydration shell. The free H_2O was found to be only about 2% of the total hydration ^1H_2Os. For equilibrium hydrated samples [e.g., ≥100% H_2O bound (w/v); Chamulitrat and Irwin, 1989], this value increased to a level >50%, whereupon there would be on the order of 10 H_2O molecules per monomer unit (Chamulitrat and Irwin, 1989). These microwave power saturation data further argue that our relaxation parameter, $\Gamma\{T_1T_2\}$, is a reasonably comparative measure of T_{1e}- which can most accurately be measured using time domain EPR techniques (Kevan and Schwartz, 1979).

Relationship between Internal Nitroxyl Amide Motions in the Wall Matrix and the Dissolution of the Cell Wall's Middle Lamella. Three experiments were performed on equivalently labeled (5.35×10^{18} spins/g ± 7% error) apple cortical tissue at two levels of ripeness (Figure 6). One treatment consisted of tissue held for 21 days at 20 °C, and the other was the control, or day 0, sample. Across all experiments, $\Gamma\{T_1T_2\}$ for the control group varied 6%, while $\Gamma\{T_1T_2\}$ for the comparison group varied only 9%; this experimental error is of the order of the variation due to least-squares estimation of our spin–

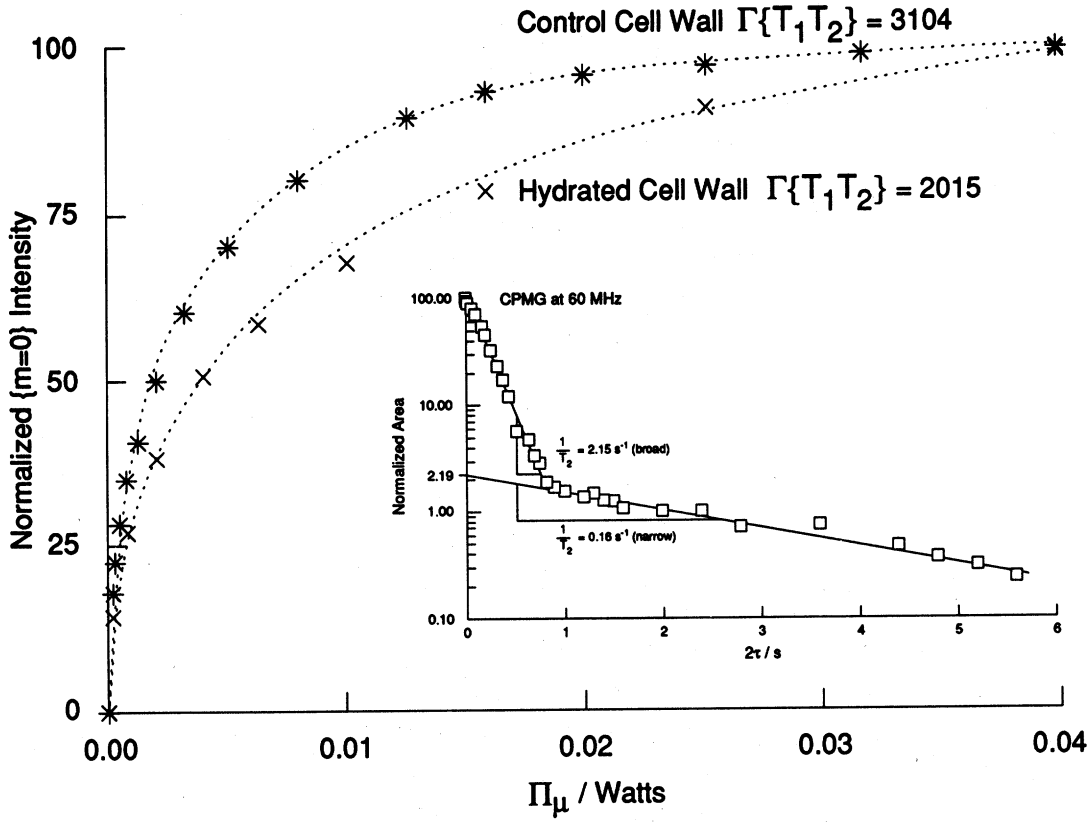

Figure 5. Partial power saturation curves of nitroxyl amide-labeled day 0 apple cell wall powders in the dry and 60% hydrated state fit to the Metz function. (Inset) Carr–Purcell–Meiboom–Gill ^{1}H NMR experiment at 60 MHz on a comparably hydrated cell wall sample in which the paramagnetic amide was reduced to the N–OH form.

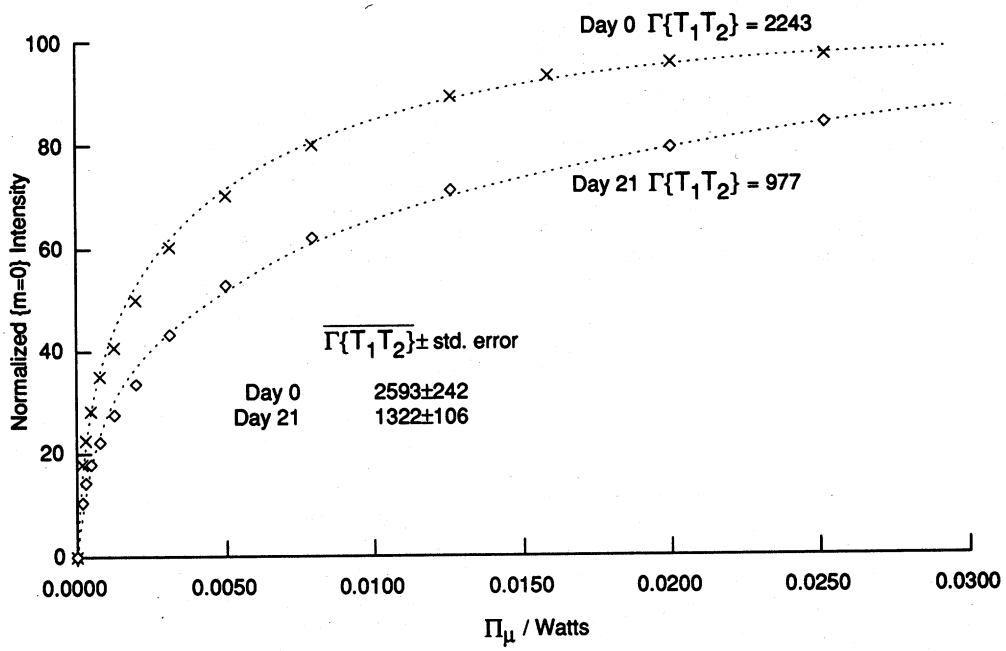

Figure 6. Partial power saturation curves of nitroxyl amide-labeled day 0 and 21 apple cell wall powders fit to the Metz function. (Inset) Results of similar power saturation experiments averaged across three experiments.

lattice relaxation parameter itself. Using the analysis of variance method (Steele and Torrie, 1960) for a randomized complete block experimental design, we found that the day 0 and day 21 treatments differed significantly from each other at the 5% confidence level.

Figure 7 summarizes experiments representing $^{23}Na^+$ T_1s, C=O $T_{1\rho}$s, and C=O-associated T_{1H}s (Irwin et al., 1984a) relative to $\Gamma\{T_1T_2\}$, as well as a comparative scale of the dissolution of the middle lamella (as measured by relative tissue rigidity), as a function of 0, 11, and 21 days at 20 °C. $^{23}Na^+$ relaxation times (Figure 7, stars) were measured on Na^+-exchanged apple cell walls. We observed a small (ca. 19%), but significant, change in $T_{1^{23}Na^+}$ as a function of these treatments, which argues that the *local* carboxyl group's environment in these cell wall polyuronides was sensitive to the perturbations occurring as a function of fruit ripening. Similar results were noted for changes (ca. 15%) in the carboxyl ^{13}C $T_{1\rho}$s (Figure 7, diamonds) which measure *local* motions in the 10–100-kHz range (Havens and Koenig, 1983). Interestingly, the

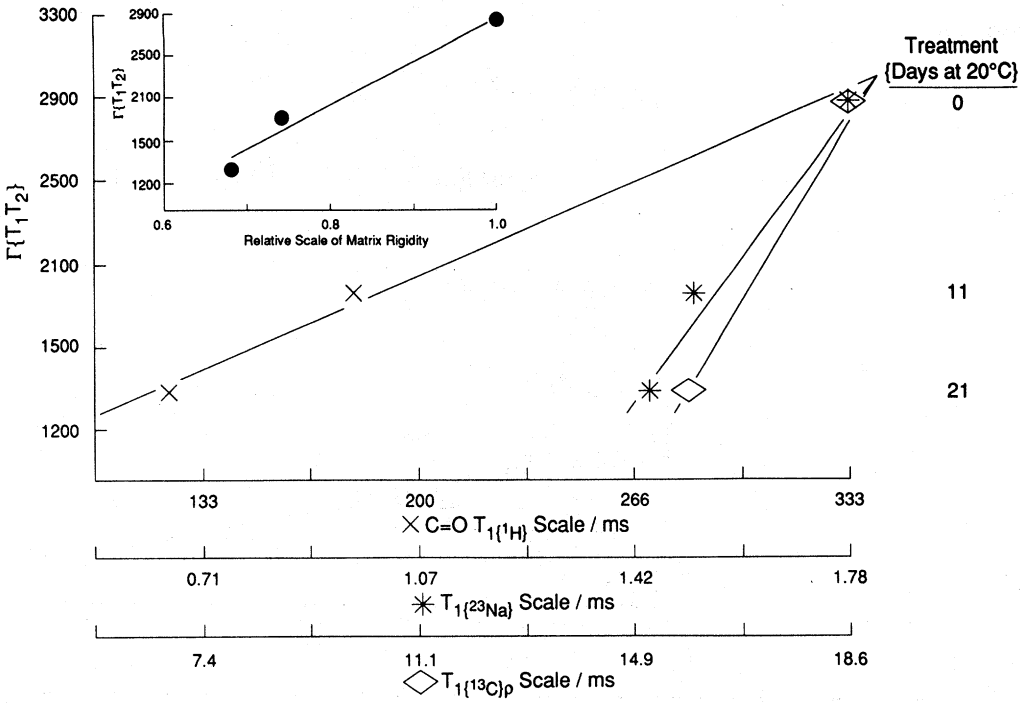

Figure 7. Plot of nitroxyl amide-labeled cell wall $\Gamma\{T_1T_2\}$s as a function of various relaxation times and other measures of wall matrix dissolution.

nitroxyl amide $\Gamma\{T_1T_2\}$s (Figure 7, *y* axis) for these identical samples changed in a linear fashion, with respect to all of the measured relaxation times, about 50% over the course of the experiment. Lastly, our supramolecular, or global, measure of pectinic polysaccharide motion was made via inversion recovery, with cross-polarization and magic angle spinning (CPMAS) NMR, for the apple cell wall's C=O resonance. In higher plant cell wall samples the C=O resonance is known to represent, as a rule, the polyuronide fraction of plant cell walls (Irwin et al., 1984a, 1985a). This indirect gauge of ^{1}H relaxation, via the observation of the C=O resonance, is important because polymeric solids rich in directly bonded ^{1}Hs, such as carbohydrates, display rapid spin diffusion which averages molecular motions across large arrays. Thus, C=O resonance ^{1}H relaxation times, via cross-polarization, are inversely proportional to the motion of the globally averaged uronide-containing polymers because the C=O resonance results from the magnetization transfer from a pool of ^{1}Hs directly bonded to cell wall components containing carboxyl functional groups. Of all of our relaxation time experiments C=O T_{1H}s were most sensitive to middle lamellar dissolution since they displayed a ca. 63% decline.

In summary, the breadth of the molecular level dynamics, as measured by various spin–lattice relaxation parameters, follow the order *local* (ca. ≤10 Å) < *internal* or *main chain* (10–100 Å; e.g., on the order of 1–10 uronic acid monomer units long) < *supramolecular* (>100 Å). Our results strongly argue that the changes in polygalacturonan mobility or flexibility during the cell separation process and dissolution of the middle lamella result from higher-order disruptions in the structure of pectin, or one of its near-neighbor matrix polysaccharides, since the greatest relative perturbation was in the proton T_1, our supramolecular measure of motion. Our data support various sources (Knee, 1978a,b, 1982; Irwin et al., 1988; Irwin, 1989) which suggest that the disruption of cohesion in apple cortical tissue results from processes *other* than the cleavage of the polyuronide backbone.

ACKNOWLEDGMENT

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LITERATURE CITED

Abragam, A. *Principles of Nuclear Magnetism*; Clarendon Press: Oxford, U.K., 1986; pp 451–474.

Bartley, I.; Knee, M.; Casimir, M. Fruit Ripening I. Changes in Cell Wall Composition and Endo-polygalacturonase in Ripening Pears. *J. Exp. Bot.* **1982**, *33*, 1248–1255.

Ben-Arie, R.; Kislev, N.; Frenkel, C. Ultrastructural Changes in the Cell Walls of Ripening Apple and Pear Fruit. *Plant Physiol.* **1979**, *64*, 197–202.

Chamulitrat, W.; Irwin, P. Homopolygalacturonan Nitroxyl Amides: Matrix Deformation Induced Motional Perturbations of Cell Wall Polyuronides. *Macromolecules* **1989**, *22*, 2685–2693.

Chamulitrat, W.; Irwin, P.; Sivieri, L.; Schwartz, R. Homopolygalacturonan Nitroxyl Amides: Hydration-Induced Motion. *Macromolecules* **1988**, *21*, 141–146.

Darvill, A.; McNeil, M.; Albersheim, P.; Delmer, D. The Primary Cell Walls of Flowering Plants. In *The Biochemistry of Plants*; Academic Press: New York, 1980; Vol. I, pp 91–162.

Davis, M.; Gidley, M.; Morris, E.; Powell, D.; Rees, D. Intermolecular Association in Pectin Solutions. *Int. J. Biol. Macromol.* **1980**, *2*, 330–332.

de Vries, J.; Voragen, A.; Rombouts, F.; Pilnik, W. Structural Studies of Apple Pectins with Pectolytic Enzymes. In *The Chemistry and Function of Pectin*; Fishman, M., Jen, J., Eds.; American Chemical Society: Washington, DC, 1986; pp 38–48.

Draper, N.; Smith, H. *Applied Regression Analysis*; Wiley: New York, 1981; pp 462–465.

Eaton, G.; Eaton, S. Electron Paramagnetic Resonance. In *Analytical Instrumentation Handbook*; Dekker: New York, 1990; pp 467–530.

Evelyn, L.; Hall, L. Nitroxide Spin-labelling of Amino and Carboxyl Groups of Monosaccharide Derivatives, Mediated by Dicyclohexylcarbodiimide. *Carbohydr. Res.* **1979**, *70*, C1.

Farrar, T. *Pulse Nuclear Magnetic Resonance Spectroscopy*; Farragut Press: Chicago, 1987; pp 53–57.

Fishman, M.; Pfeffer, P.; Barford, R.; Doner, L. Studies of Pectin Solution Properties by High Performance Size Exclusion Chromatography. *J. Agric. Food Chem.* **1984**, *32*, 372–378.

Fishman, M.; Pepper, L.; Damert, W.; Phillips, J.; Barford, R. A Critical Reexaminatioin of Molecular Weight and Dimensions for Citrus Pectin. In *The Chemistry and Function of Pectin*; Fishman, M., Jen, J., Eds.; American Chemical Society: Washington, DC, 1986; pp 22–37.

Havens, J.; Koenig, J. Applications of High-resolution Carbon-13 Nuclear Magnetic Resonance Spectroscopy to Solid Polymers. *Appl. Spectrosc.* **1983**, *37*, 226–249.

Irwin, P. The Structure and Degradation of Intact Plant Cell Wall Matrices by ^{13}C CPMAS NMR and Related Techniques. In *Nuclear Magnetic Resonance in Agriculture*; Pfeffer, P., Gerasimowicz, W., Eds.; CRC Press: Boca Raton, 1989; pp 337–354.

Irwin, P.; Pfeffer, P.; Gerasimowicz, W.; Pressey, R.; Sams, C. Ripening-related Perturbations in Apple Cell Wall Nuclear Spin Dynamics. *Phytochemistry* **1984a**, *23*, 2239–2242.

Irwin, P.; Sevilla, M.; Shieh, J. ESR Evidence for Sequential Divalent Cation Binding in Higher Plant Cell Walls. *Biochim. Biophys. Acta* **1984b**, *805*, 186–190.

Irwin, P.; Gerasimowicz, W.; Pfeffer, P.; Fishman, M. ^{1}H–^{13}C Polarization Transfer Studies of Uronic Acid Polymer Systems. *J. Agric. Food Chem.* **1985a**, *33*, 1197–1201.

Irwin, P.; Sevilla, M.; Stoudt, C. ESR Spectroscopic Evidence for Hydration- and Temperature-dependent Spatial Perturbations of a Higher Plant Cell Wall Paramagnetic Ion Lattice. *Biochim. Biophys. Acta* **1985b**, *842*, 76–83.

Irwin, P.; Sevilla, M.; Osman, S. Spectroscopic Evidence for Spatially Sequential Amide Bond Formation in Plant Homopolygalacturonans. *Macromolecules* **1987**, *20*, 1222–1227.

Irwin, P.; Sevilla, M.; Chamulitrat, W. Homopolygalacturonan Molecular Size in Plant Cell Wall Matrices via Paramagnetic Ion and Nitroxyl Amide Dipolar Spin-spin Interactions. *Biophys. J.* **1988**, *54*, 337–344.

Irwin, P.; Sevilla, M.; Chamulitrat, W.; Hoffman, A. Nitroxyl Amide Spin Labeling: Methyl Esterification-, Hydration- and Ca^{2+}-Induced Motional Perturbations of Pectinic Polysaccharides in Apples. In *Physical Chemistry of Food Processes*; Van Nostrand Reinhold: New York, 1992; Vol. II, in press.

Jarvis, M. Structure and Properties of Pectin Gels in Plant Cell Walls. *Plant Cell Environ.* **1984**, *7*, 153–164.

Kevan, L.; Schwartz, R. Modulation of Electron Spin-Echo Decay in Solids. In *Time Domain Electron Spin Resonance*; Kevan, L., Schwartz, R., Eds.; Wiley: New York, 1979; pp 279–341.

Knee, M. Properties of Polygalacturonate and Cell Cohesion in Apple Fruit Cortical Tissue. *Phytochemistry* **1978a**, *17*, 1257–1260.

Knee, M. Metabolism of Polymethylgalacturonate in Apple Fruit Cortical Tissue During Ripening. *Phytochemistry* **1978b**, *17*, 1261–1264.

Knee, M. Fruit Softening II. Precursor Incorporation into Pectin by Pear Tissue Slices. *J. Exp. Bot.* **1982a**, *33*, 1256–1262.

Knee, M. Fruit Softening. III. Requirement for Oxygen and pH Effects. *J. Exp. Bot.* **1982b**, *33*, 1263–1269.

Matthys, P.; Callens, F.; Boesman, E. An Algorithm for the Fitting of EPR Steady-state Saturation Curves. *Phys. Status Solidi B* **1988**, *146*, K149–K151.

Metz, H.; Völkel, G.; Windsch, W. A Simple Method for Fitting of Inhomogeneous ESR Saturation Curves. *Phys. Status Solidi A* **1990**, *122*, K73–K76.

O'Beirne, D.; Van Buren, J. Size Distribution of High Weight Species in Pectin Fractions from Idared Apples. *J. Food Sci.* **1983**, *48*, 276–277.

Pake, G.; Estle, T. The Phenomenon of Magnetic Resonance. In *The Physical Principles of Electron Paramagnetic Resonance*, 2nd ed.; Pines, D., Ed.; Benjamin: Reading, MA, 1973; pp 1–57.

Poole, C. Relaxation Times. In *Electron Spin Resonance, A Comprehensive Treatise on Experimental Techniques*, 2nd ed.; Wiley: New York, 1983; pp 577–643.

Preston, R. In *The Physical Architecture of Plant Cell Walls*; Chapman and Hall: London, 1974; pp 1–15.

Renard, C.; Searle van Leeuwen, M.; Voragen, A.; Thibault, J.-F.; Pilnik, W. Studies on Apple Protopectin. II: Apple Cell Wall Degradation by Pure Polysaccharides and their Combinations. *Carbohydr. Polym.* **1991**, *14*, 295–314.

Schaefer, J.; Stejskal, E. High-resolution ^{13}C NMR of Solid Polymers. In *Topics in C-13 NMR Spectroscopy*; Wiley: New York, 1979; Vol. III, pp 283–324.

Steele, R.; Torrie, J. Analysis of Variance II: Multiway Classifications. In *Principles and Procedures of Statistics*; McGraw-Hill: New York, 1960; pp 132–160.

Taylor, R.; Conrad, H. Stoichiometric Depolymerization of Polyuronides and Glycosaminoglycuronans to Monosaccharides Following Reduction of Their Carbodiimide-activated Carboxyl Groups. *Biochemistry* **1972**, *11*, 1383–1388.

Wertz, J.; Bolton, J. Time-dependent Phenomena. In *Electron Spin Resonance, Elementary Theory and Practicle Applications*; Chapman and Hall: New York, 1986; pp 192–222.

Yannoni, C. High-Resolution NMR in Solids: The CPMAS Experiment. *Acc. Chem. Res.* **1982**, *15*, 201–208.

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